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TLR Signaling Is Required for *Salmonella typhimurium* Virulence

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SUMMARY

Toll-like receptors (TLRs) contribute to host resistance to microbial pathogens and can drive the evolution of virulence mechanisms. We have examined the relationship between host resistance and pathogen virulence using mice with a functional allele of the *nramp-1* gene and lacking combinations of TLRs. Mice deficient in both TLR2 and TLR4 were highly susceptible to the intracellular bacterial pathogen *Salmonella typhimurium*, consistent with reduced innate immune function. However, mice lacking additional TLRs involved in *S. typhimurium* recognition were less susceptible to infection. In these TLR-deficient cells, bacteria failed to upregulate *Salmonella* pathogenicity island 2 (SPI-2) genes and did not form a replicative compartment. We demonstrate that TLR signaling enhances the rate of acidification of the *Salmonella*-containing phagosome, and inhibition of this acidification prevents SPI-2 induction. Our results indicate that *S. typhimurium* requires cues from the innate immune system to regulate virulence genes necessary for intracellular survival, growth, and systemic infection.

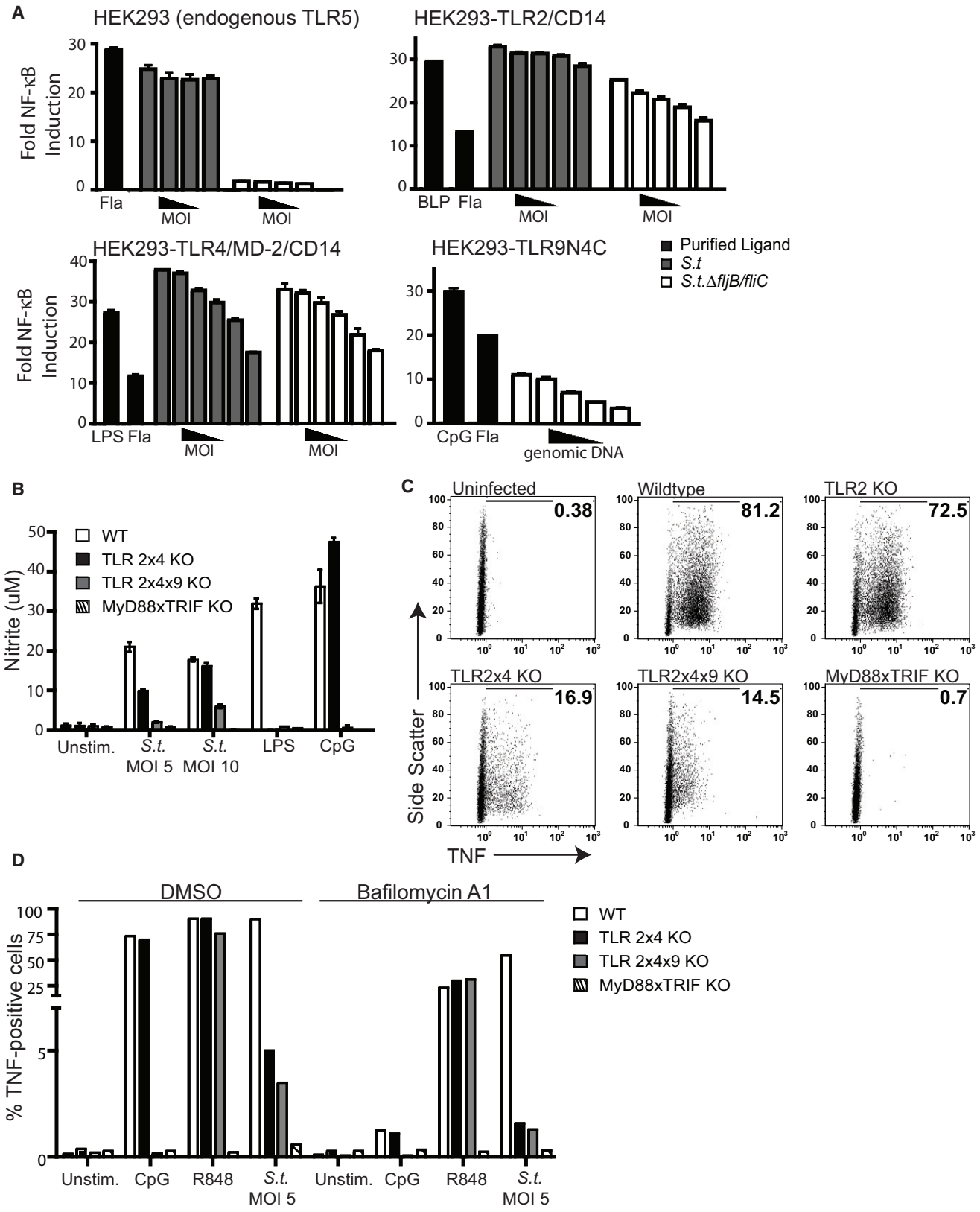
INTRODUCTION

During early stages of infection the innate immune system is essential for limiting microbial replication and spread before an adaptive response is mounted. Accordingly, pathogens have evolved virulence strategies to antagonize innate immune function (Hedrick, 2004; Rausher, 2001; Woolhouse et al., 2002). The interplay between host innate immune function and pathogen virulence mechanisms largely determines the outcome of most infections. Despite the logic of this conceptual framework, our understanding of the molecular interactions driving the emergence of virulence mechanisms remains relatively poor.

Innate immune receptors detect infection by recognizing conserved microbial features common to broad classes of microbes (Janeway, 1989; Medzhitov, 2007). The Toll-like receptors (TLRs) target a range of microbial ligands, including lipopolysaccharide (TLR4), lipoproteins (TLR2), flagellin (TLR5), unmethylated CpG motifs in DNA (TLR9), double-stranded RNA (TLR3), and single-stranded RNA (TLR7 and TLR8) (Akira et al., 2001; Kawai and Akira, 2005). Expression of TLRs on innate immune cells links microbial recognition to induction of antimicrobial mechanisms, such as production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and expression of antimicrobial peptides (AMPs). In addition, TLR activation can promote adaptive immunity through control of dendritic cell (DC) maturation (Iwasaki and Medzhitov, 2004).

To study the evolution of pathogen virulence and its relationship to innate immunity, we have focused on TLR-mediated recognition of *Salmonella enterica* serovar *typhimurium*. *S. typhimurium* is a gram-negative bacterium that can survive and replicate within host macrophages (Coburn et al., 2007). Survival within macrophages requires a set of genes, many of which are encoded within *Salmonella* pathogenicity island 2 (SPI-2) (Galan, 2001; Shea et al., 1996; Waterman and Holden, 2003). SPI-2 encodes a type 3 secretion system (T3SS) that is expressed after the bacterium is phagocytosed (Cirillo et al., 1998; Pfeifer et al., 1999; Valdivia and Falkow, 1997). Translocation of SPI-2 effectors into the host cell transforms the phagosome into a compartment that supports bacterial replication, the *Salmonella*-containing vacuole (SCV) (Marcus et al., 2000). Multiple signals have been implicated in the transcriptional induction of SPI-2, including cation deprivation, phosphate starvation, and low pH (Chakravortty et al., 2005; Cirillo et al., 1998; Deiwick et al., 1999; Kim and Falkow, 2004; Rappl et al., 2003). Most of the studies implicating these signals have been performed on bacteria grown in vitro; whether the same signals are responsible for induction of SPI-2 genes within the phagosome remains unclear.

Recognition of *S. typhimurium* is largely mediated by TLR2, TLR4, and TLR5 (Feuillet et al., 2006; Hapfelmeier et al., 2005; O'Brien et al., 1980; Royle et al., 2003; Smith et al., 2003; Uematsu et al., 2006; Vazquez-Torres et al., 2004). Consistent with a central role for these receptors, *S. typhimurium* has evolved mechanisms to subvert this recognition or to avoid the consequences of TLR activation. For example, modification of


A
HEK293 (endogenous TLR5)
HEK293-TLR2/CD14
HEK293-TLR4/MD-2/CD14
HEK293-TLR9N4C
Fold NF-κB Induction
Fla
BLP Fla
LPS Fla
CpG Fla
MOI
genomic DNA
Purified Ligand
S.t
S.t.ΔfljB/fliC
B
Nitrite (uM)
WT
TLR 2x4 KO
TLR 2x4x9 KO
MyD88xTRIF KO
Unstim.
S.t. MOI 5
S.t. MOI 10
LPS
CpG
C
Uninfected
Wildtype
TLR2 KO
TLR2x4 KO
TLR2x4x9 KO
MyD88xTRIF KO
0.38
81.2
72.5
16.9
14.5
0.7
Side Scatter
TNF
D
DMSO
Bafilomycin A1
% TNF-positive cells
WT
TLR 2x4 KO
TLR 2x4x9 KO
MyD88xTRIF KO
Unstim.
CpG
R848
S.t. MOI 5

lipid A by pagP reduces recognition by TLR4, although this modification is probably most relevant for resistance to AMPs (Bader et al., 2005; Detweiler et al., 2003; Guo et al., 1997, 1998). Mice lacking TLRs, especially TLR4, are more susceptible to *S. typhimurium* (Weiss et al., 2004). To circumvent the problem of redundancy, many studies have used mice lacking the common TLR adaptor MyD88 or lacking both MyD88 and another adaptor, TRIF (Hapfelmeier et al., 2005; Weiss et al., 2004). Although these mice are very susceptible to *S. typhimurium*, these studies suffer from the caveat that MyD88 is also required for signaling by members of the IL-1 receptor (IL-1R) family. Because mice deficient in IL-1R are more susceptible to infection, the phenotype of MyD88 knockout (KO) mice cannot be unequivocally attributed to TLRs (Mayer-Barber et al., 2010; Raupach et al., 2006).

In the studies described here, we sought to eliminate TLR-based recognition of *S. typhimurium* and examine the effect on pathogen virulence, while avoiding the caveats associated with MyD88-KO mice. In addition, we were concerned that the extreme susceptibility of C57Bl/6 mice (the genetic background on which most studies with TLR-KO mice have been performed) to *S. typhimurium* infection might mask any relationships between TLRs and bacterial virulence strategies. Many inbred mouse strains, including C57Bl/6, possess a nonfunctional allele of the *nramp-1* gene. *nramp-1* encodes a multipass transmembrane protein that localizes to lysosomes and functions as a transporter of divalent cations, and mice with the nonfunctional allele are extremely susceptible to a number of intracellular pathogens (Forbes and Gros, 2001; Govoni et al., 1996; Vidal et al., 1993, 1995, 1996).

To avoid the caveats associated with nonfunctional Nramp-1 and TLR-independent functions of MyD88, we generated mice with a functional allele of *nramp1* that lack individual or multiple TLRs. Studies in these mice led to a striking finding. Whereas mice lacking a subset of the TLRs involved in *S. typhimurium* recognition showed increased susceptibility to infection, a lack of additional TLRs resulted in reduced susceptibility. The loss of virulence correlated with an inability of bacteria to survive and replicate within macrophages. We show that TLR signaling leads to rapid acidification of the SCV, and this signal is required for regulation of virulence gene expression. In the absence of this contextual cue, *S. typhimurium* is unable to survive and replicate intracellularly. Altogether, this work describes the molecular interactions underlying a bacterial pathogen's dependence on the innate immune system for virulence.

RESULTS

Multiple TLRs Are Involved in Recognition of *S. typhimurium*

To identify which TLRs are relevant for innate recognition of *S. typhimurium*, we utilized HEK293 reporter cell lines expressing an NF-κB-luciferase reporter construct. Stimulation of these cells with heat-killed bacteria resulted in robust induction of NF-κB, which we attributed to endogenous TLR5 expressed by these cells (Figure 1A). This response was abrogated when cells were stimulated with bacteria lacking flagellin. To measure activation of other TLR family members, HEK293 reporter cells stably expressing individual TLRs were stimulated with bacteria lacking flagellin (to eliminate the contribution of endogenous TLR5). Using this approach, we observed activation of TLR2 and TLR4 by *S. typhimurium* (Figure 1A). Furthermore, *S. typhimurium* genomic DNA was capable of activating a surface-localized version of TLR9 (Figure 1A).

Although these results confirmed that TLR2, TLR4, TLR5, and TLR9 may play a role in recognition of *S. typhimurium*, they did not address the relative importance of each TLR during infection. To this end, we infected bone marrow-derived macrophages (BMMs) lacking combinations of TLRs and measured production of nitric oxide (NO). In agreement with previously published studies, BMMs lacking both TLR2 and TLR4 (TLR2×4-KO) produced much less NO than wild-type BMMs (Figure 1B). The remaining response was partially dependent on TLR9, as BMMs lacking TLR2, TLR4, and TLR9 (TLR2×4×9-KO) produced even less NO. Similar results were observed when tumor necrosis factor alpha (TNF) production was measured (Figure 1C). Importantly, all genotypes of BMMs responded equivalently to the TLR7 ligand R848, indicating that the cells were otherwise equivalent (Figure 1D and Figure S1 available online). The small amount of TNF and NO produced in TLR2×4×9-KO BMMs was dependent on other TLRs, as BMMs lacking both MyD88 and TRIF (and therefore all TLR-dependent signaling) did not respond to *S. typhimurium* (Figures 1B and 1C). As TLR5 is not expressed in murine BMMs, we reasoned that the residual TNF and NO produced by TLR2×4×9-KO BMMs was most likely due to TLR7 or TLR3 signaling. To address this possibility directly, we pretreated TLR2×4×9-KO BMMs with bafilomycinA1, an inhibitor of the vacuolar ATPase (V-ATPase) that prevents activation of endosomal TLRs. BafilomycinA1 treatment inhibited TNF production in TLR2×4-KO and TLR2×4×9-KO BMMs to almost background

Figure 1. Multiple TLRs Recognize Products of *S. typhimurium*

(A) HEK293 cells expressing the indicated TLRs or TLR accessory proteins together with an NF-κB luciferase reporter were treated with heat-killed wild-type LT2 *S. typhimurium* (*S.t.*), flagellin-deficient LT2 (*S.t.*Δ*fljB*/*fliC*), or genomic DNA isolated from flagellin-deficient LT2. Luciferase activity was measured after 8 hr. Relative moi range: 100 to 3.125, DNA concentration: 375 ng/ml to 12.5 ng/ml. Data are representative of two independent experiments and shown as mean ± standard error of the mean (SEM). LPS, lipopolysaccharide; Fla, flagellin; BLP, bacterial lipopeptide.

(B) BMMs differentiated from the indicated mice were treated overnight with 100 U/ml recombinant IFN-γ and infected the next morning with wild-type *S. typhimurium* (SL1344) at the indicated moi. Nitrite production was measured 36 hr post-infection by Griess assay. Data are representative of three independent experiments and presented as mean ± standard deviation (SD).

(C) BMMs were infected as in (B) at an moi of 5 for 8 hr, followed by intracellular cytokine staining for TNF. Percent TNF-positive cells are indicated in each panel. Data are representative of three independent experiments.

(D) BMMs pretreated for 2 hr with bafilomycinA1 or DMSO vehicle were infected with *S. typhimurium* (SL1344) at an moi of 5 for 6 hr. Cells were processed and stained for intracellular cytokine staining as in (C).

levels, suggesting that TLR7 and/or TLR3 are responsible for the remaining TNF production in response to *S. typhimurium* (Figure 1D). Collectively, these data indicate that TLR2, TLR4, TLR9, and TLR7 (and/or TLR3) each contribute to the recognition of *S. typhimurium* in infected BMMs.

TLR Signaling Is Required for *S. typhimurium* Virulence

Having established which TLRs respond to ligands derived from *S. typhiumurium* in BMMs, we sought to test the effect of TLR deficiency on bacterial virulence in vivo. We crossed a functional allele of the *nramp1* gene onto the C57BL/6 background and generated TLR-deficient or TLR-adaptor-deficient mice with functional Nramp1 (see Extended Experimental Procedures). We expected that reduced TLR function would lead to greater susceptibility to infection. Indeed, all TLR2×4-KO mice died within 16 days when challenged orally with *S. typhimurium*, whereas 75% of the wild-type mice survived for the duration of the experiment (Figure 2A). By contrast, TLR2×4×9-KO mice were *less* susceptible to infection than TLR2×4-KO mice, despite a greater impairment in TLR function (Figure 2A). This increased survival was not a consequence of reduced immunopathology due to reduced TLR function. In fact, TLR2×4×9-KO mice had lower numbers of bacteria 4 days post-infection in spleens, livers, ceca, and mesenteric lymph nodes (MLNs) relative to TLR2×4-KO mice (Figure 2B). Thus, despite less robust innate immune function, *S. typhimurium* was less virulent in TLR2×4×9-KO mice.

The difference in susceptibility between TLR2×4-KO and TLR2×4×9-KO mice could indicate that TLR9 plays a negative role in immunity to *S. typhimurium*. To test this possibility, we challenged mice lacking TLR4 and TLR9 (TLR4×9-KO). We reasoned that if TLR9 were playing a negative role in immunity, then any genotype lacking TLR9 would be resistant to infection. Instead, TLR4×9-KO mice were as susceptible to infection as TLR2×4-KO mice, indicating that lack of TLR9 by itself does not confer increased resistance to infection (Figure 2A). Thus, the data presented suggest that overall TLR signaling is in some way required for *S. typhimurium* virulence. Despite this apparent requirement, MyD88-KO and MyD88×TRIF-KO mice (with wild-type Nramp1) were highly susceptible to *S. typhimurium* infection (Figure S2). As discussed earlier, the extreme sensitivity of these mice relative to TLR2×4×9-KO mice is likely due to the role of MyD88 downstream of the IL-1, IL-18, and IL-33 receptors (Mayer-Barber et al., 2010; Raupach et al., 2006). Thus, to examine the role for TLR signaling in *S. typhimurium* virulence, we must use TLR-deficient mice, not mice lacking common signaling adaptors.

One potential caveat of these in vivo studies is that the commensal flora may be different between TLR2×4-KO and TLR2×4×9-KO mice. Recent studies have reported alterations in commensal communities in mice lacking certain TLRs or TLR-signaling adaptors (Vijay-Kumar et al., 2008; Wen et al., 2008). To address this possibility, we challenged mice with a different gram-negative enteric pathogen, *Yersinia enterocolitica* (*Y. enterocolitica*), which shares a similar route of intestinal colonization but remains extracellular after crossing the intestinal epithelia. In contrast to our experiments with *S. typhimurium*, TLR2×4×9-KO mice were equally, if not more, susceptible relative to TLR2×4-KO mice (Figure 2C). The differential sensitivity of TLR2×4×9-KO mice to these two enteric bacteria argues that alterations in commensal flora are not contributing to the phenotypes of TLR2×4-KO and TLR2×4×9-KO mice. Instead, the reduction in TLR signaling in TLR2×4×9-KO mice appears to specifically impact the virulence of *S. typhimurium*.

TLR Signaling Is Required for Intracellular Growth of Bacteria

Because survival within macrophages is required for systemic infection (Fields et al., 1986; Leung and Finlay, 1991), we next used a gentamicin protection assay to examine survival and replication in BMMs lacking various TLRs. Consistent with our in vivo experiments, *S. typhimurium* was able to replicate in TLR2×4-KO but not TLR2×4×9-KO BMMs (Figure 3A). When we counted bacteria in individual BMMs by immunofluorescence microscopy (IF), the number of bacteria per cell in TLR2×4-KO BMMs accumulated over time, whereas the number of bacteria per cell in TLR2×4×9-KO BMMs remained constant, indicating that bacterial replication was responsible for the differences in colony-forming units (CFU) between genotypes (Figures 3B and 3C). We observed a similar lack of bacterial replication in MyD88×TRIF-KO BMMs (Figure 3A). Unlike our in vivo experiments, the phenotype of MyD88×TRIF-KO BMMs is most likely due to a deficiency in TLR signaling, as the IL-1 receptor family is not involved in the initial recognition of *S. typhimurium* within BMMs in vitro. Furthermore, TLR4×9-KO BMMs supported bacterial replication similarly to TLR2×4-KO BMMs, corroborating the conclusions from our in vivo experiments (Figures 3B and 3C). TLR2×4×9-KO and MyD88×TRIF-KO BMMs did support replication of *Listeria monocytogenes* and *Legionella pneumophila* (Figure S3). In addition, *S. typhimurium* replicated well in MyD88×TRIF-KO BMMs lacking functional Nramp1 (Figure S3). These results indicate that phagosomes of TLR-deficient cells are formally capable of supporting bacterial growth, but the combination of functional Nramp1 and lack of TLR signaling prevents *S. typhimurium* replication.

Collectively, these data suggest that *S. typhimurium* requires TLR signaling for replication in macrophages. However, the lack of replication in wild-type BMMs would appear to contradict this conclusion, as TLR function is normal in these cells. When we counted the number of bacteria per cell by IF, though, we observed a similar increase in bacteria per cell over time as in TLR2×4-KO BMMs (Figure 3C). This contradiction was resolved when we measured cell death of BMMs after infection. Wild-type BMMs exhibited greater cell death relative to each of the other genotypes (Figures 3D and 3E). Because only wild-type BMMs express functional TLR4, the increased death of these cells seems likely to be due to a previously described TLR4-dependent cell death that occurs in *S. typhimurium* infected cells (Cook et al., 2007; Hsu et al., 2004; Park et al., 2002). Thus, the apparent lack of replication as measured by CFU in wild-type BMMs is the result of macrophage death followed by gentamicin-mediated killing of the bacteria. In contrast, the inability of *S. typhimurium* to replicate in TLR2×4×9-KO or MyD88×TRIF-KO BMMs is due to a different mechanism.

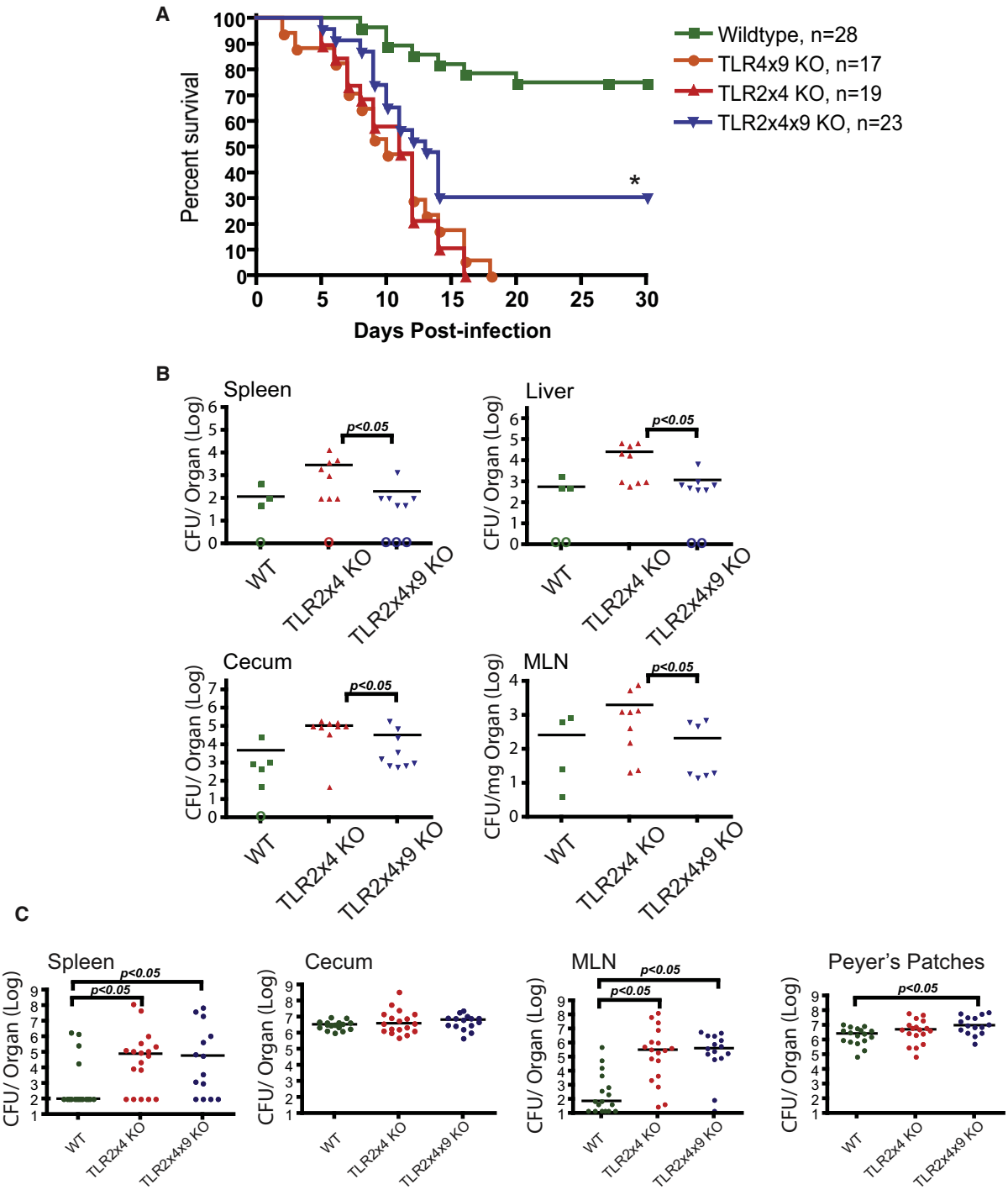


Figure 2. TLR2×4×9-KO Mice Are Less Susceptible to *S. typhimurium* than TLR2×4-KO Mice

(A) Survival plots of mice orally inoculated with 1.6×10^8 CFU *S. typhimurium* (SL1344) are shown. *$p < 0.005$ by log-rank curve comparison test. Data are representative of at least two independent experiments.

(B) Groups of 8- to 10-week-old mice were orally inoculated with 1×10^9 CFU *S. typhimurium* (SL1344). Four days post-infection organs were harvested and homogenized for colony enumeration. Data are representative of at least three independent experiments.

(C) Groups of mice of the indicated genotype were orally inoculated with 2×10^8 *Y. enterocolitica* and CFU were measured in the indicated organs 3 days post-infection. Data presented are the combined results from two independent experiments.

For (B) and (C), bars represent mean CFU of all mice, with data significance determined by Mann-Whitney U test. Open circles indicate mice for which no colonies were detected. MLN, mesenteric lymph node. See also Figure S2.

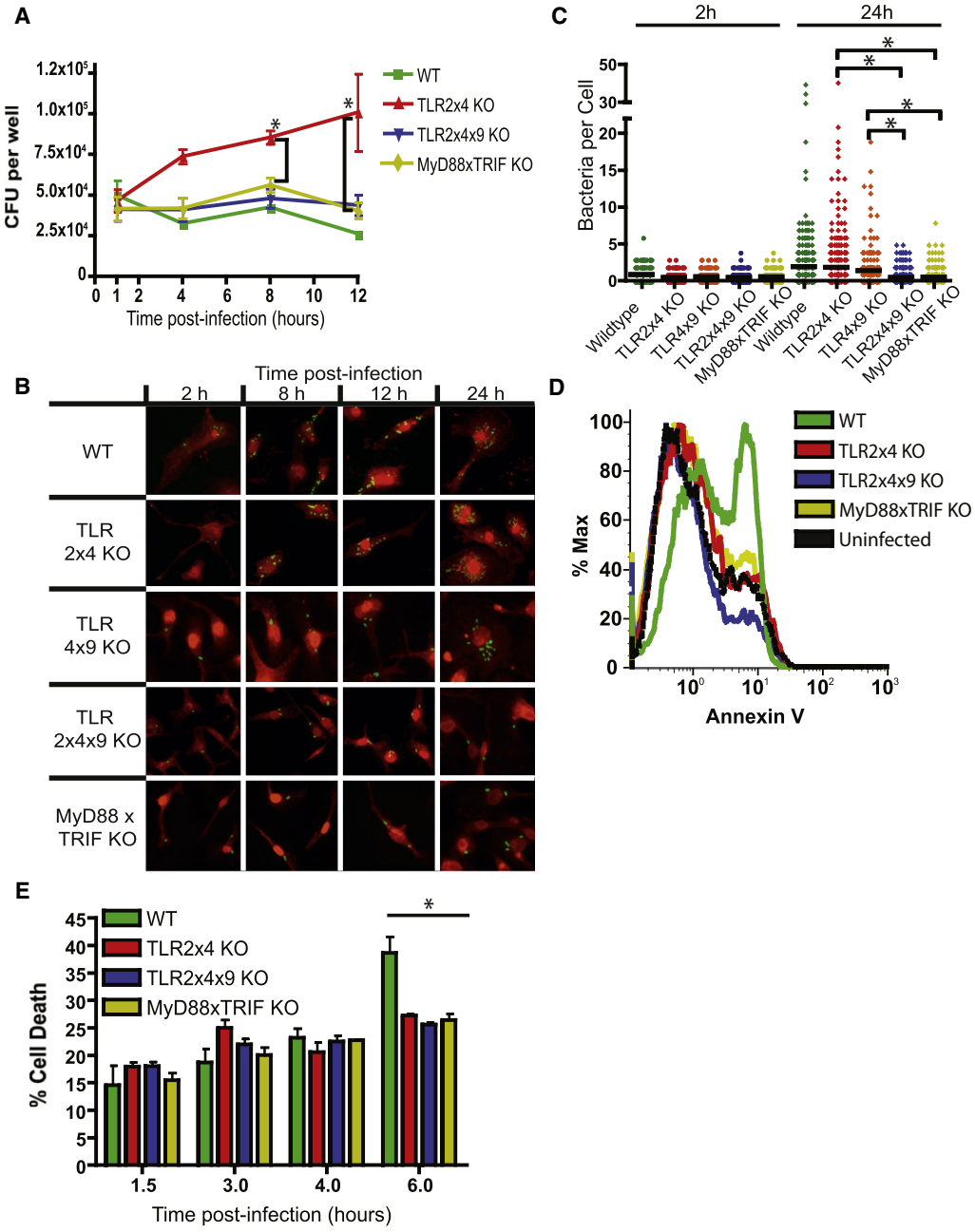


Figure 3. *S. typhimurium* Is Unable to Replicate in TLR2×4×9-KO and MyD88×TRIF-KO BMMs

(A) BMMs derived from mice of the indicated genotypes were infected with *S. typhimurium* (SL1344) at an moi of 1, and intracellular CFU were measured by a gentamicin protection assay at the indicated time points. Data are presented as the average of three independent experiments ± SEM. *$p < 0.05$ by student's t test comparing TLR2×4-KO to MyD88×TRIF-KO and TLR2×4×9-KO.

(B) BMMs infected as described in (A) were fixed and permeablized at the indicated times post-infection followed by staining with anti-*Salmonella* LPS antibody (green) and wheat germ agglutinin (red).

(C) Intracellular bacteria per cell were counted in random fields at the 2 and 24 hr time points from Z-stacked images as shown in (B). Data are representative of two independent experiments, p value determined by student's t test (*$p < 0.05$).

(D) BMMs of the indicated genotypes were infected with *S. typhimurium* (SL1344) at an moi of 5. Eight hours post-infection, cells were harvested and stained with Annexin V. Data are representative of two independent experiments.

(E) BMMs were infected at an moi of 10 with *S. typhimurium* (SL1344), and release of lactate dehydrogenase (LDH) was measured in supernatants at the indicated time points. Data are presented as mean ± SD and are representative of at least two independent experiments; p value determined by student's t test (*$p < 0.05$) comparing wild-type to all other genotypes.

See also Figure S3.

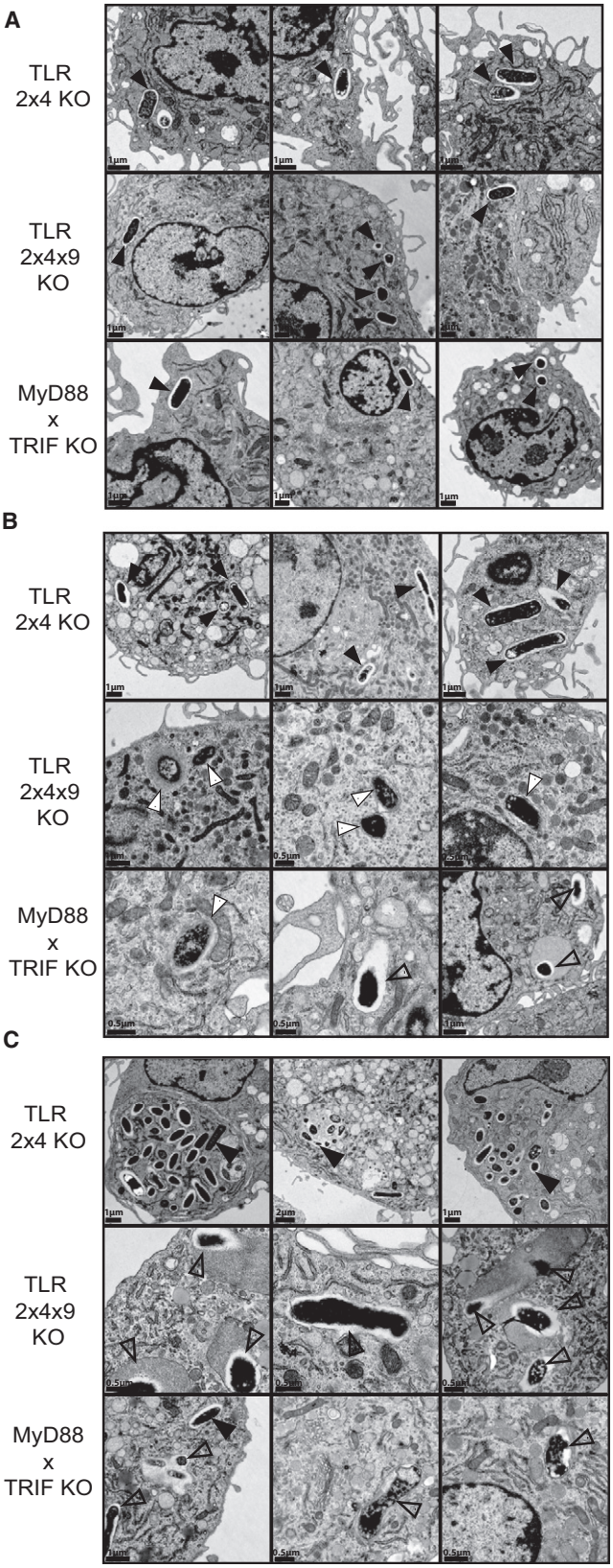


Figure 4. *S. typhimurium* Fails to Form an SCV in TLR2×4×9-KO and MyD88×TRIF-KO BMMs

TLR2×4-KO, TLR2×4×9-KO, or MyD88×TRIF-KO BMMs were infected at an moi of 10 with *S. typhimurium* (SL1344), and cells were fixed and processed for electron microscopy at 2 hr (A), 8 hr (B), and 22 hr (C) post-infection. Bacteria in intact vacuoles are shown with filled black arrowheads, cytosolic bacteria with filled white arrowheads, and bacteria that are degraded or have fused with lytic compartments are indicated with open black arrowheads. Micron bars are in the lower left corner of each panel. Three representative images are shown from different sections and independent infections.

TLR Signaling Is Required for Establishment of the SCV

To better understand why *S. typhimurium* is unable to replicate in TLR2×4×9-KO and MyD88×TRIF-KO BMMs, we used transmission electron microscopy (EM) to investigate the fate of bacteria in infected BMMs. At 2 hr post-infection, bacteria were clearly visible in well-defined vacuoles in BMMs of all three genotypes (Figure 4A, black triangles). By 8 hr and 22 hr post-infection, bacteria in TLR2×4-KO BMMs remained largely unchanged, although evidence of replication was evident, especially at 22 hr (Figures 4B and 4C). In contrast, phagosomes containing bacteria in TLR2×4×9-KO and MyD88×TRIF-KO BMMs were quite distinct. The bacteria often appeared mottled or irregular in shape, and in many cases bacteria were surrounded by electron-dense staining material consistent with lysosomal fusion (Figure 4, open triangles). In some instances, bacteria were no longer surrounded by membrane, suggesting that they entered the cytosol (Figure 4, white triangles). Cytosolic bacteria have been described when bacteria fail to secrete certain SPI-2 effectors (Beuzon et al., 2000). In total, the images clearly demonstrate a defect in the ability of *S. typhimurium* to establish a replicative compartment in TLR2×4×9-KO and MyD88×TRIF-KO BMMs.

Induction of SPI-2 Genes by TLR Signaling

Our studies thus far indicate that intracellular growth of *S. typhimurium* is impaired in TLR2×4×9-KO and MyD88×TRIF-KO BMMs and suggest that this defect may be related to inefficient SCV formation. We next sought to define the underlying basis for impaired growth in BMMs lacking TLR function by profiling gene expression of bacteria isolated from BMMs of each genotype. To overcome the lack of sensitivity of microarray-based approaches, we performed quantitative RT-PCR to measure expression of all genes in the *S. typhimurium* genome (Figure 5A).

Using K-means clustering analysis, we identified subsets of genes with differential expression profiles between the BMM genotypes. Genes within the SPI-2 locus were upregulated in bacteria in wild-type and TLR2×4-KO BMMs but not in bacteria in TLR2×4×9-KO or MyD88×TRIF-KO BMMs. For validation, we reanalyzed expression of each gene within the SPI-2 locus and adjacent to the locus (as controls), using independent RNA samples from infected BMMs of all genotypes. As shown in Figure 5B, 13 genes within the SPI-2 locus were upregulated in wild-type and TLR2×4-KO BMMs but not in TLR2×4×9-KO and MyD88×TRIF-KO BMMs. These 13 genes most likely underestimate the extent to which the entire SPI-2 locus is differentially expressed between BMM genotypes, as many genes were statistically excluded due to extremely low levels of message

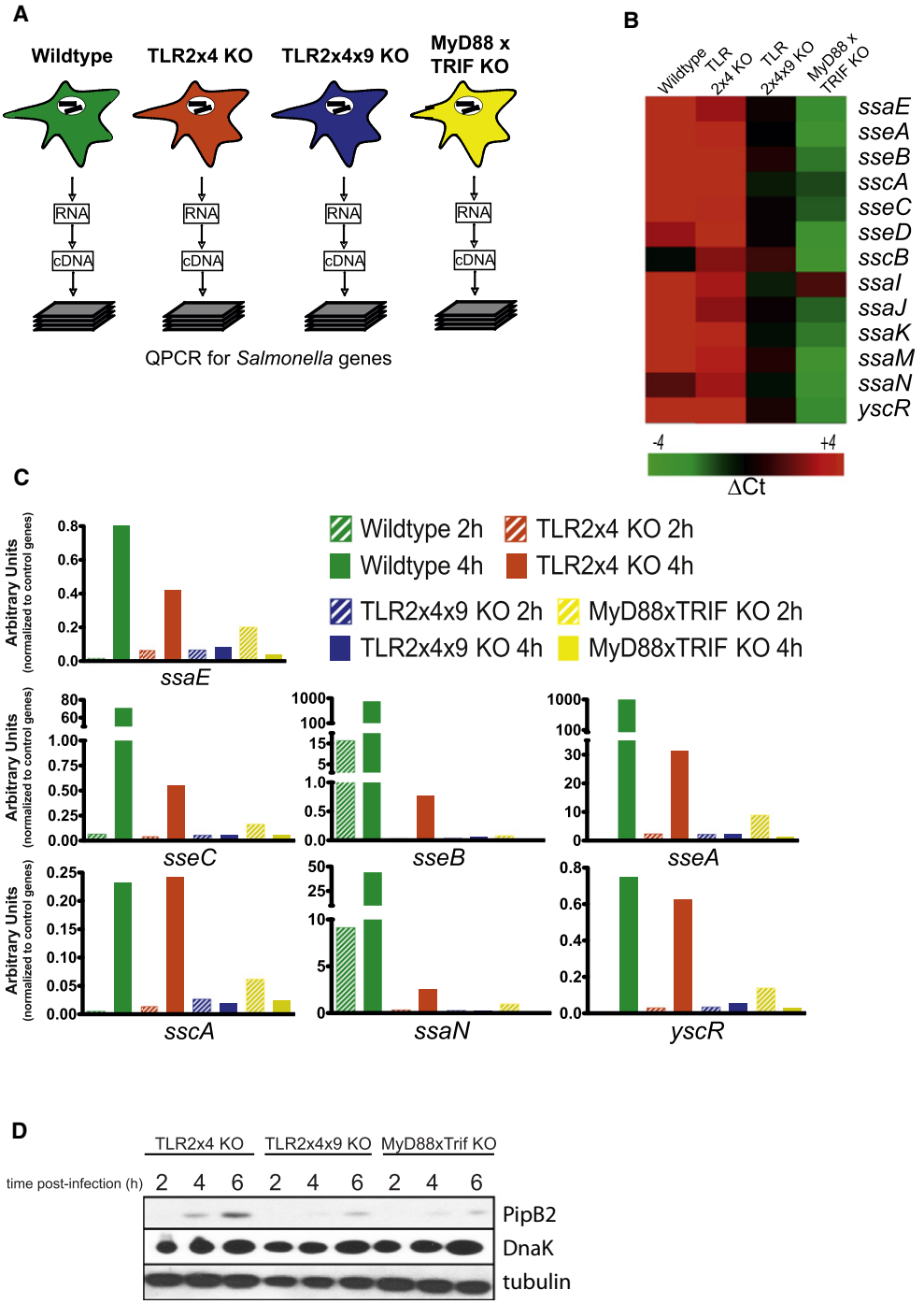


Figure 5. *S. typhimurium* Fails to Upregulate and Secrete SPI-2 Effectors in TLR2×4×9-KO and MyD88×TRIF-KO BMMs

(A) Schematic of quantitative expression analyses for *S. typhimurium* genes. Total RNA was isolated from infected BMMs of the indicated genotypes followed by processing for quantitative RT-PCR (see Experimental Procedures).

(B) Heat map of normalized expression data for SPI-2 genes in bacteria within BMMs of the indicated genotypes. For each BMM genotype, data are shown relative to the 2 hr time point. The gene designation is to the right of each row.

(C) Relative induction of individual SPI-2 genes in bacteria isolated from BMMs of the indicated genotypes. Data are normalized to the average expression values of a set of control genes. The data presented in (B) and (C) represent the mean of two independent experiments.

(D) BMMs of the indicated genotypes were infected with an *S. typhimurium* strain (12032) expressing an HA-tagged allele of *pipB2* expressed from the endogenous *pipB2* locus. The presence of PipB2 in BMM lysates was detected by immunoprecipitation and immunoblot with anti-HA antibodies. Controls for numbers of BMM (tubulin) and bacteria (DnaK) are also shown. Data are representative of three independent experiments.

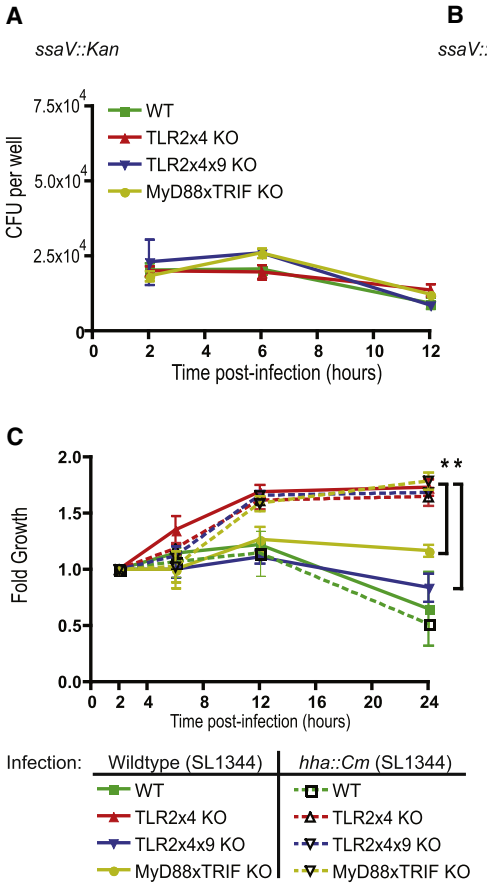


Figure 6. TLR Signaling Is Necessary for SPI-2 Induction and Intracellular Growth

(A) BMMs of the indicated genotypes were infected (moi of 1) with SPI-2 mutant *S. typhimurium* (SL1344 *ssaV::Kan*). Intracellular CFU were measured by a gentamicin protection assay.

(B) TLR2×4-KO BMMs were infected as in (A) followed by fixation and processing for electron microscopy. Bacteria in intact vacuoles are indicated by filled black triangles, cytosolic bacteria by filled white triangles, and bacteria that are degraded or have fused with lytic compartments by open black triangles. Three representative images from different sections and from independent infections are shown.

(C) BMMs of the indicated genotypes were infected (moi of 1) with wild-type *S. typhimurium* or a strain with constitutive SPI-2 expression (SL1344 *hha::Cm*). Intracellular CFU were measured via a gentamicin protection assay at the indicated time points post-infection. Data are presented as mean fold over the first time point (to control for minor inoculum differences between strains) ± SEM and are representative of three independent experiments. *p < 0.05 by student's t test comparing TLR2×4-KO to MyD88×TRIF-KO and TLR2×4×9-KO at the indicated time point.

in TLR2×4×9-KO or MyD88×TRIF-KO samples. For most SPI-2 genes, induction was higher in wild-type BMMs relative to TLR2×4-KO BMMs (Figure 5C), suggesting that induction correlates with the strength of TLR signaling. Thus, the lack of intracellular replication in TLR-deficient cells may be due to a failure to upregulate SPI-2 genes.

These expression-profiling studies indicated that transcription of SPI-2 genes within BMMs depends on signals downstream of TLR activation. To view SPI-2 induction at the protein level, we utilized a strain of *S. typhimurium* (12023) with an HA-tagged allele of *pipB2*, a SPI-2 effector. 12023 displays the same dependence on TLR signaling for intracellular growth as SL1344 (Figure S3D). PipB2 was strongly induced and secreted in infected TLR2×4-KO BMMs (Figure 5D). In contrast, the levels of PipB2 were significantly reduced in TLR2×4×9-KO BMMs and barely detectable in MyD88×TRIF-KO BMMs, despite equivalent numbers of bacteria in all samples (indicated by DnaK levels). These data are consistent with our transcriptional analyses and indicate that TLR signaling is required for the induction of SPI-2 genes.

SPI-2 Genes Are Required for Intracellular Growth

We hypothesized that the impaired induction of SPI-2 genes in bacteria isolated from TLR2×4×9-KO and MyD88×TRIF-KO BMMs was responsible for the defect in SCV formation and intracellular replication in these cells. To test this hypothesis, we compared the fates of bacteria lacking a functional SPI-2 secretion system (*ssaV::Kan*) in BMMs of each genotype. As expected, SPI-2 mutant bacteria were unable to replicate in BMMs of any genotype (Figure 6A). Moreover, EM analysis of SPI-2 mutant bacteria in TLR2×4-KO BMMs revealed the same lack of SCV formation observed for wild-type bacteria in TLR2×4×9-KO and MyD88×TRIF-KO BMMs (Figure 6B).

If the lack of intracellular growth in TLR2×4×9-KO and MyD88×TRIF-KO BMMs is due to failure to induce SPI-2 genes, then an *S. typhimurium* strain with constitutive expression of SPI-2 genes should regain the ability to grow in these cells. To test this possibility directly, we constructed a strain lacking *hha* (Δ*hha, hha::Cm*), a negative regulator of SPI-2 genes. Previous work has demonstrated that the Δ*hha* mutant strain expresses SPI-2 genes constitutively (Silphaduang et al., 2007). Remarkably, Δ*hha* mutant bacteria replicated equivalently in BMMs of all genotypes, except wild-type cells where the lack of growth is due to TLR4-dependent cell death (Figure 6C). Although Hha probably negatively regulates additional *S. typhimurium* virulence genes, restoration of growth in TLR-deficient BMMs is consistent with the conclusion that constitutive expression of SPI-2 genes can bypass the requirement for TLR signaling.

Induction of SPI-2 Genes Requires TLR-Dependent Acidification of the SCV

Our results thus far indicate that TLR signaling provides a cue used by *S. typhimurium* to regulate SPI-2 expression. TLR activation induces host transcription as well as more proximal

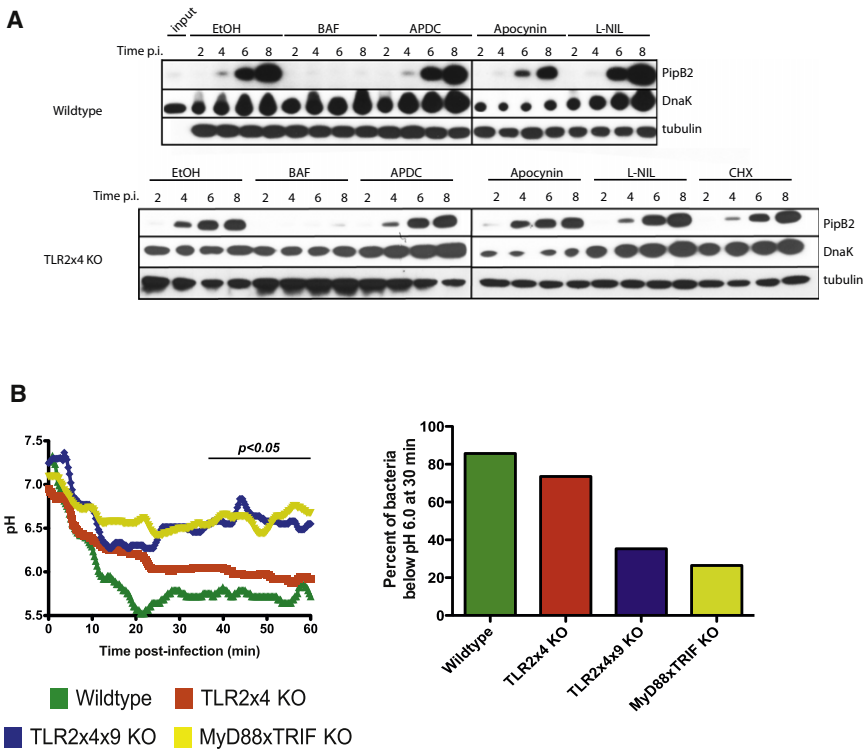


Figure 7. TLR-Dependent Phagosomal Acidification Is Required for SPI-2 Expression

(A) BMMs pretreated with each of the indicated inhibitors were infected with an *S. typhimurium* strain (12032) expressing an HA-tagged allele of *pipB2*. PipB2 levels in BMM lysates were detected by immunoprecipitation and immunoblot with anti-HA antibodies at 2, 4, 6, and 8 hr post-infection. Immunoblots for tubulin and DnaK serve as loading controls for BMMs and bacteria, respectively. Data are representative of two independent experiments for wild-type cells and three independent experiments for TLR2×4-KO BMMs.

(B) BMMs were infected with FITC-labeled *S. typhimurium* (SL1344), and the fluorescence intensities of individual bacteria excited at 490 nm or 440 nm were measured over time by live-cell imaging. The fluorescence intensity ratio (490/440) reflects the pH within the phagosome (see Experimental Procedures). The plots presented represent the mean pH calculated from at least 35 independent bacteria in multiple imaging fields. The right panel shows the percent of bacteria at or below pH 6 at 30 min post-infection.

See Experimental Procedures and Figure S4.

effects, such as production of ROS and RNS and phagosome maturation and acidification, although this last aspect remains controversial. Using pharmacological inhibitors to block each of these potential signals we measured the effect on PipB2 induction and secretion. Treatment of TLR2×4-KO BMMs with cyclohexamide (CHX) had no effect on PipB2 induction, indicating that host translation was not required for generation of the signal sensed by *S. typhimurium* (Figure 7A, bottom panel). Similarly, blocking ROS or RNS production did not prevent PipB2 induction. However, inhibition of the V-ATPase with bafilomycinA1 blocked *S. typhimurium* induction of PipB2 in both TLR2×4-KO and wild-type BMMs. The block in TLR2×4-KO cells could be due to an inhibition of TLR signaling, as bafilomycinA1 almost completely inhibits the residual response to *S. typhimurium* (Figure 1D). In wild-type cells, though, TLR2 and TLR4 signaling is largely unaffected by bafilomycinA1, suggesting that TLR-dependent acidification of the SCV may be the signal required by *S. typhimurium* for SPI-2 gene induction (Figure 7A, top panel). Experiments analyzing the induction of SPI-2 genes at the transcriptional level also indicated a requirement for phagosome acidification (data not shown).

Based on these data, we hypothesized that the lack of SPI-2 induction in TLR2×4×9-KO and MyD88×TRIF-KO BMMs is due to failure of SCVs to acidify. The issue of whether TLR signaling influences the kinetics of phagosomal maturation remains controversial (Blander and Medzhitov, 2004, 2006a, 2006b; Russell and Yates, 2007; Yates and Russell, 2005). To investigate this issue, we used ratiometric imaging to measure the pH of *Salmonella*-containing phagosomes in BMMs of each genotype. Whereas the mean pH of SCVs in wild-type and TLR2×4-KO BMMs dropped below 6 within 60 min post-infection, SCVs in TLR2×4×9-KO and MyD88×TRIF-KO BMMs failed to acidify to the same extent and exhibited slower acidification kinetics (Figure 7B). By 30 min post-infection, over 70% of SCVs in wild-type and TLR2×4-KO BMMs had reached pH 6, whereas less than 35% of SCVs in TLR2×4×9-KO and MyD88×TRIF-KO BMMs had similarly acidified (Figure 7B). Consistent with the lower transcriptional induction of SPI-2 genes in TLR2×4-KO BMMs (relative to wild-type), the rate of acidification in TLR2×4-KO cells was slower than in wild-type cells, despite ultimately reaching pH 6 by 60 min. Collectively, these data support a model in which TLR signaling accelerates phagosomal acidification, which is used by *S. typhimurium* as a cue for SPI-2 gene induction.

DISCUSSION

Biological interactions are strong drivers of evolution, and the dynamics of host-pathogen interactions provide some of the clearest examples of this principle. Hosts have evolved resistance mechanisms, such as TLRs, that work by reducing pathogen fitness and drive the evolution of pathogen virulence (Hedrick, 2004; Rausher, 2001; Woolhouse et al., 2002). Although virulence genes provide a fitness advantage, they can be energetically costly and often serve as targets of host sensors (Miao et al., 2010; Vance et al., 2009). Therefore, the ability to regulate expression of virulence genes based on changing environments is a key feature of microbial pathogenesis. In this study, we report the requirement of TLR signaling for *S. typhimurium* to establish a successful infection and cause disease. We demonstrate that this requirement stems, at least in part, from the need for TLR-dependent phagosome acidification

to induce SPI-2 genes, resulting in replication and virulence of the microbe. These data demonstrate that a pathogen can evolve to require innate immune signaling for full virulence.

Previous studies have demonstrated that host genetic variation can result in prolonged survival upon infection (Raberg et al., 2007). However, these phenotypes are generally attributable to reduced inflammation and immunopathology, suggesting that the host is more tolerant to an increased pathogen burden (Gowen et al., 2006; Wang et al., 2004). By contrast, our work demonstrates that mice lacking sufficient TLR signaling are less susceptible to an *S. typhimurium* infection due to reduced bacterial growth. A similar relationship has been described in *Drosophila*, where mutations in the melanization arm of the innate immune response render flies less susceptible to *Streptococcus pneumoniae* with reduced levels of bacteria, but the mechanism behind this observation remains unclear (Ayres and Schneider, 2008). Our work clearly shows that *S. typhimurium* fails to induce virulence genes when deprived of innate immune signals.

Crosstalk between Nramp-1 and TLR Signaling

Two aspects of our approach were crucial for our ability to observe the requirement for TLR-dependent signals in *Salmonella* virulence. First, by using mice that are deficient in multiple TLRs, as opposed to mice lacking MyD88 and TRIF, we were able to circumvent the susceptibility associated with lack of IL-1R family function. Indeed, the difference that we observe in susceptibility between MyD88×TRIF-KO and TLR2×4×9-KO mice underscores the importance of the IL-1R family in defense against infection. We were somewhat surprised by the role for nucleic acid-sensing TLRs in innate recognition of *Salmonella*, although TLR9 and TLR7 have been implicated in recognition of bacterial nucleic acid (Bafica et al., 2005; Mancuso et al., 2009). Although the simplest explanation for this observation is that some bacteria are degraded, it is also possible that a nucleic acid ligand is secreted by *Salmonella* or present on the bacterial surface (Whitchurch et al., 2002; Woodward et al., 2010).

A second critical aspect of our study is that we used mice with a functional allele of *nramp1*. Why the lack of this protein renders mice so susceptible to intracellular pathogens remains unclear, but this heightened sensitivity may simply obviate any requirement for TLR-dependent SPI-2 induction. The presence of functional Nramp1 has been shown to enhance SPI-2 expression as well as TLR-dependent responses (Fritsche et al., 2003; Valdez et al., 2008; Zaharik et al., 2002). Regardless of the precise mechanism responsible for the strong TLR dependence when Nramp1 is functional, it is important to recognize that infection of cells with functional Nramp1 represents the "wild-type" scenario. Indeed, mutations in the human Nramp1 gene are associated with increased susceptibility to several intracellular pathogens (Bellamy et al., 1998; Malik et al., 2005). Therefore, examining virulence in the presence of functional Nramp1 most accurately reflects the host-pathogen interactions between *S. typhimurium* and the mammalian immune system.

TLR Signaling Alters the pH of the *Salmonella*-Containing Vacuole

Our studies indicate that the difference in susceptibility of TLR-deficient mice is due to lack (or substantial delay) of SPI-2 induction. We show that SPI-2 induction requires phagosome acidification, and our measurements of phagosomal pH indicate that acidification is impaired and/or delayed in TLR-deficient cells. The extent to which TLR signaling influences phagosomal maturation (including increasing phagolysosomal fusion, acidification, and proteolytic activity) has remained a contentious issue (Blander and Medzhitov, 2004, 2006a, 2006b; Russell and Yates, 2007; Yates and Russell, 2005). Although our studies were not designed to address this controversy, we clearly show that TLR signaling is required for rapid acidification of the SCV and has profound implications for the fate of intracellular bacteria and disease outcome. The mechanism is likely similar to the acidification of lysosomes during DC maturation, when TLR signaling leads to recruitment of the V1 subunit of the vacuolar ATPase to the lysosomal membrane (Trombetta et al., 2003). The precise signaling pathways that lead to assembly of this machinery are unknown. Moreover, whether bacteria sense pH directly or utilize other phagosomal features that require acidic pH remains unclear.

Importantly, we are not suggesting that phagosome maturation cannot occur without TLR signaling. Indeed, our EM images of infected TLR2×4×9-KO and MyD88×TRIF-KO BMMs at late time points show bacteria within electron-dense compartments, suggestive of phagolysosomal fusion. Due to technical limitations, we have not extended our pH measurements beyond 60 min post-infection, but our images suggest that phagosomes in TLR-deficient cells eventually mature. In fact, we do observe a small percentage of SCV in TLR2×4×9-KO and MyD88×TRIF-KO cells with significant reductions in pH within 1 hr (Figure S4). The lack of bacterial replication in TLR-deficient cells suggests that the eventual maturation of phagosomes is not sufficient to induce SPI-2 genes or that the induction occurs too late to prevent bacterial killing by lysosomal contents. It is also possible that the phagosome breaks down in the absence of SPI-2 function, and bacteria enter the cytosol where they are unable to replicate (Beuzon et al., 2000). Our EM analyses suggest that both of these possibilities may contribute to the lack of bacterial replication.

Innate Immune Signaling as an Environmental Cue for Virulence Gene Regulation

These findings have important implications for our understanding of the evolution of host-pathogen interactions and virulence mechanisms. Many pathogens trigger these mechanisms by utilizing signals downstream of innate receptors, most likely as a reliable mechanism to properly induce genes necessary for survival in the presence of antimicrobial mechanisms. For example, the PhoP/PhoQ two-component system, when activated by AMPs, induces expression of genes that modify lipidA and render the bacterial membrane more resistant to AMPs (Bader et al., 2005; Guo et al., 1998). By a potentially similar mechanism, prior activation of cells with TLR ligands can increase replication of *S. typhimurium* (Wong et al., 2009). Our finding that *S. typhimurium* has evolved to require host-resistance signals for proper expression of virulence genes is conceptually distinct from these previously described antagonistic strategies. Notably, *S. typhimurium* is unable to replicate in TLR-deficient cells, despite the absence of the antimicrobial

mechanisms normally induced by TLRs. One implication of this remaining dependence is that the virulence genes induced by TLR signaling are required for purposes other than simply evading TLR-induced antimicrobial mechanisms to promote *S. typhimurium* fitness.

Why would *Salmonella* use signals downstream of TLRs to broadly coordinate expression of virulence genes required for intracellular growth? These signals may be the most reliable contextual cues that *Salmonella* can use to sense its presence within a macrophage phagosome. In general, a fundamental problem faced by *Salmonella* is the need to interact with multiple cell types through the course of an infection. Unique sets of virulence genes are required to survive each of these stages, and *Salmonella* must recognize its environment and induce the appropriate genes. For example, *Salmonella* must detect when it has encountered a macrophage and induce SPI-2 genes, which are necessary for formation of the SCV and maintenance of the integrity of the phagosome. Precise regulation of such virulence genes is clearly essential for optimal growth, as mutant bacteria with constitutive expression of SPI-2 genes (e.g., *Δhha* mutants) are attenuated in vivo (Coombes et al., 2005; Silphaduang et al., 2007). Inappropriate expression of certain virulence genes could result in decreased fitness due to recognition by innate sensors or may disrupt proper regulation of other virulence genes required at specific stages of infection. Therefore, *Salmonella* utilizes TLR-dependent signals within the phagosome to detect its presence within a macrophage. Linking the induction of virulence genes (including SPI-2) to phagosomal signals downstream of TLRs may be an efficient way of coordinating multiple virulence mechanisms in response to a unifying contextual cue.

EXPERIMENTAL PROCEDURES

Cell Culture

BMMs were differentiated from bone marrow for 5 days using macrophage colony-stimulating factor (M-CSF) as previously described (Ewald et al., 2008). See Extended Experimental Procedures for details.

Bacterial Strains and Infections

Overnight cultures of *S. typhimurium* were opsonized for infection. BMMs were spin-infected, incubated at 37°C, then washed with PBS before the addition of 10 μg/ml gentamicin media. For intracellular CFU determination, cells were washed with PBS and lysed in 1% Triton X-100 in PBS. Lysates were plated on LB agar plates containing 200 μg/ml streptomycin (Life Technologies). See Extended Experimental Procedures for strain details and descriptions of assays with *L. monocytogenes* and *L. pneumophila*.

Measurement of Cell Death

Lactate dehydrogenase (LDH) release was quantified using the CytoTox 96 Nonradioactive cytotoxicity kit (Promega) according to manufacturer's instructions. Annexin V staining was performed using Annexin V-FITC (BD Pharmingen) in Annexin V staining buffer according to manufacturer's instructions.

Measurement of BMM Activation

NO was quantified in supernatants from BMMs treated overnight with 100 U/ml recombinant IFN-γ (R&D Systems) using the Griess assay (all reagents from Sigma Aldrich). TNF-α production was measured by intracellular cytokine staining using anti-TNF-α antibody (eBioscience) according to manufacturer's instructions (eBioscience). All steps prior to fixation were performed in the presence of 10 μg/ml gentamicin. Cells were analyzed on an FC500 flow cytometer (Beckman Coulter).

S. typhimurium Effector Secretion

BMMs were infected (multiplicity of infection [moi] of 25) with *S. typhimurium pipB2*-2xHA (12032). At the indicated time points, cells were washed with PBS and lysed in 1% NP-40 in PBS with protease-inhibtor cocktail (Roche) and EDTA (Fisher), and lysates were subjected to immunoprecipitation with rat anti-HA agarose beads (Roche). Cells were pretreated with inhibitors for 1 hr before infection. See Extended Experimental Procedures for a more detailed explanation of sample processing and detection of effector secretion.

Mice and In Vivo Infections

All animal experiments were performed in accordance with University of California Animal Care and Use Committee guidelines. See Extended Experimental Procedures for descriptions of strains and backcrossing analyses. For survival and CFU enumeration experiments, age-matched mice were fasted for 14 hr followed by oral gavage with 100 μl *S. typhimurium* (SL1344) or *Yersinia enterocolitica* (8081) in PBS (see figure legends for CFU). For CFU enumeration, organs were harvested, homogenized in PBS using a Polytron PT2100 homogenizer (Kinematica), diluted, and plated on streptomycin (for *Salmonella*) or irgasan (1 μg/ml) (for *Yersinia*) LB-agar plates.

Gene Expression Analyses

RNA from infected BMMs (moi of 5) was extracted with Trizol RNA reagent, purified using PureLink Micro-to-Midi total RNA purification system, DNase-treated, reverse transcribed using random hexamers (all reagents from Life Technologies), and processed for quantitative PCR. Sample processing, primer sequences/design, and description of data analysis can be found in the Extended Experimental Procedures and Table S1.

Microscopy

For IF, BMMs on coverslips were stained with FITC-conjugated mouse anti-*Salmonella* antibody (clone 1E6, Santa Cruz Biotechnology) and Cy3-conjugated wheat germ agglutinin (Life Technologies) at the indicated time points. Cells were imaged on a Nikon E800 fluorescent microscope and bacteria per cell were counted in random, blinded, Z-stacked images.

For pH determination and video microscopy, BMMs plated on 4-chamber slides (Nunc) were infected with FITC-labeled bacteria. Following infection, chambers were incubated at 37°C for 5 min, washed extensively with PBS, incubated with phenol-free DMEM containing 10 μg/ml gentamicin, then visualized on a Nikon TE2000 inverted fluorescent microscope with environmental control. Images were collected with excitation at both 440 nm and 490 nm and analyzed using Imaris Scientific 3D/4D image processing and analysis software (Bitplane) to track individual intracellular bacteria. Background-subtracted fluorescence intensity values were used to determine the 490/440 ratios for each bacterium at each time point. Absolute pH values were determined by generating a standard curve using buffered pH solutions (see Extended Experimental Procedures).

For EM studies, cells were infected (moi of 10) with wild-type or *ssaV::Kan* SL1344 (as described above). At each time point, cells were fixed, embedded, sectioned, and stained for EM (see Extended Experimental Procedures).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, four figures, and one table and can be found with this article online at doi:10.1016/j.cell.2011.01.031.

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